

AWARD NUMBER: W81XWH-16-1-0461

TITLE: Novel Targeted Therapies for Inflammatory Breast Cancer

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REPORT DATE: October 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE October 2017			2. REPORT TYPE Annual		3. DATES COVERED 30 Sep 2016 - 29 Sep 2017
4. TITLE AND SUBTITLE Novel Targeted Therapies for Inflammatory Breast Cancer			5a. CONTRACT NUMBER		5b. GRANT NUMBER W81XWH-16-1-0461
			5c. PROGRAM ELEMENT NUMBER		5d. PROJECT NUMBER
6. AUTHOR(S) JOSE SILVA E-Mail: jose.silva@mssm.edu			5e. TASK NUMBER		5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Mount Sinai School of Medicine, 1 Gustave L. Levy Pl, New York, NY 10029			8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSOR/MONITOR'S ACRONYM(S)		11. SPONSOR/MONITOR'S REPORT NUMBER(S)
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Inflammatory breast cancer (IBC, ~5% of all breast cancers) is the most lethal form of breast cancer, presenting a 5-year survival rate that is less than half of the non-IBC patients. Remarkably, we have found that survival of IBC cells depends on histone deacetylase 6 (HDAC6) function. Here, first, we used these state-of-the-art system biology approaches to evaluate the response to ACY-1215 of a large series of breast cancer cells (sensitive and resistance) to identify critical hubs associated with resistance to HDAC6 inhibition. Through our studies we have found that STAT3 signaling is strongly upregulated in resistant cell lines upon inhibition HDAC6 suggesting an adaptative survival mechanism of the treated cells. Importantly STAT3 inhibitors (such as Ruxolitinib) already exist and can be easily translated to the clinic. Thus, our studies identified STAT3 inhibition as the prime candidate to synergistically interact with Ricolinostat. Additionally to STAT3, other pathways such as P38, TGF- β , and AKT has also emerged as MRs.					
15. SUBJECT TERMS Synergistic treatment, STAT3, IBC treatment, HDAC6.					
16. SECURITY CLASSIFICATION OF: a. REPORT Unclassified			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 9	19a. NAME OF RESPONSIBLE PERSON USAMRMC
b. ABSTRACT Unclassified			19b. TELEPHONE NUMBER (include area code)		
c. THIS PAGE Unclassified					

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Progress Report 1st year

1-Introduction

Inflammatory breast cancer (IBC, ~5% of all breast cancers) is the most lethal form of breast cancer, presenting a 5-year survival rate that is less than half of the non-IBC patients. Despite these facts, IBC remains poorly understood and systemic disease management relies exclusively on chemotherapy. Remarkably, we have found that survival of IBC cells depends on histone deacetylase 6 (HDAC6) function, whereas HDAC6 is mainly dispensable in non-IBCs¹. Importantly, we have demonstrated that the leading HDAC6 inhibitor (Ricolinostat, Acetylon Inc.), which is being tested in clinical trials for other tumor types, inhibits the growth of IBC cells *in vitro* and *in vivo*. Our findings represent an exciting opportunity to develop novel targeted therapies for IBC patients.

2-Keywords

Inflammatory breast cancer, targeted therapy, HDAC6 inhibitor, Ricolinostat, Ruxolitinib.

3-Accomplishments

During the past period of support we have:

- Task 1) Investigate HSP-90, DNAJB12 and MEAF6 as HDAC6 substrates that critically regulate the viability of IBC cells: Despite its canonical roles in protein in proteostasis HDAC6 could act through other unrelated substrates. Through our collaboration with Acetylon, we have identified several novel putative substrates of HDAC6. HSP-90, DNAJB12 and MEAF6 were identified as the top candidates. Thus, we are utilizing a genetic screening strategy to investigate the involvement of these genes in the lethal phenotype induced by HDAC6 inhibitors. We have already generated a CRISPR sgRNA library containing 10 guides for each of the selected genes and an additional set of 10 negative controls. This library has been used to perform genetic screens *in vitro* using the SUM-149 cell line. This screen has also been completed (Fig 1) and currently we are waiting for the sequencing results of the screen.

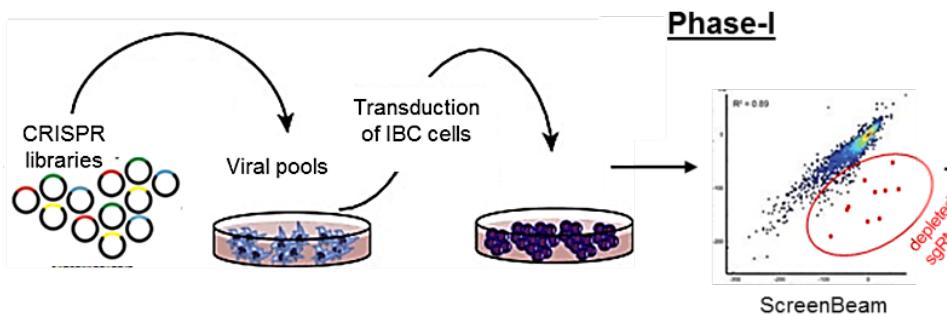


Figure 1. Graphic representation of the completed phase-I of the genetic screen to Investigate HSP-90, DNAJB12 and MEAF6 as HDAC6 substrates that critically regulate the viability of IBC cells

- Task 2.1a and 2.1b Design and evaluation of combination therapy with HDAC6 inhibition for IBC treatment. We have pioneered the development of computational and experimental methods for identifying important hub/Master Regulators (MRs) of cancer cells. These MRs represent critical gene and pathways that modulate both cell viability and response to treatments. Thus, these methods allow us to rationally select tumor targets as novel anticancer treatment as well as new therapeutic combinations. Here, first, we used these state-of-the-art

system biology approaches to evaluate the response to ACY-1215 of a large series of breast cancer cells (sensitive and resistance) to identify critical hubs associated with resistance to HDAC6 inhibition.

Our studies have identified a series of breast cancer cell lines (~10%) that are sensitive ($IC_{50} > 2.5 \mu M$) to HDAC6 inhibitors as well as a series (~50%) that are complete resistant ($IC_{50} > 7 \mu M$) to these treatment (Fig. 2), with the rest of the cell models somewhere in between. Interestingly, we found that HDAC6 function was a MR only for responsive cell lines and that these lines were enriched in hormone receptor positive and Her2 positive features (Fig. 2).

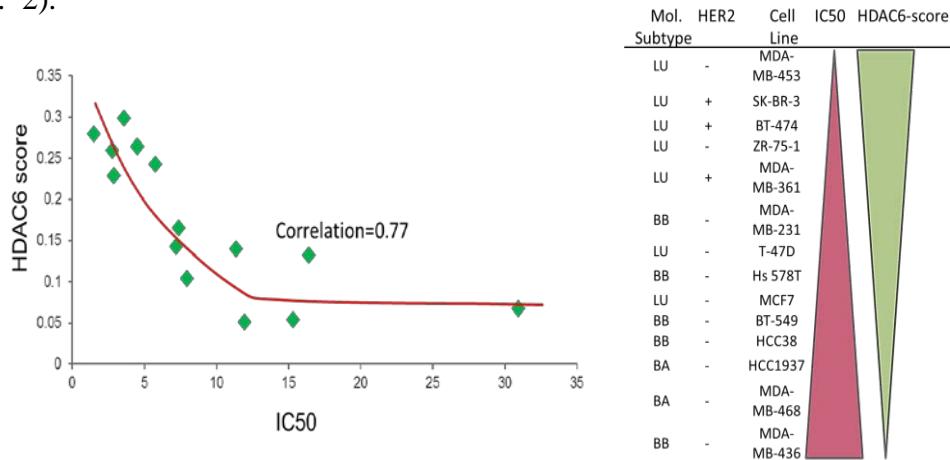


Fig. 2. MR analysis of HDAC6 response. Illustrative example of MR analysis (HDAC6 score) of selected primary breast cancer samples. The left panel shows the strong association between HDAC6-score and the response to the leading HDAC6 inhibitor Ricolinostat. The right panel summarizes the result and the molecular subtype of the breast cancer lines analyzed.

Performing comparison MR analysis between the resistant and resistant cell lines we have found that STAT3 signaling is strongly upregulated in resistant cell lines upon inhibition HDAC6 suggesting an adaptative survival mechanism of the treated cells. Importantly stat3 inhibitors (such as ruxolitinib) already exist and can be easily translated to the clinic. Thus, our studies identified STAT3 inhibition as the prime candidate to synergistically interact with Ricolinostat. Additionally to STAT3, other pathways such as P38, TGF- β , and AKT has also emerged as MRs. During the next period of support, these pathways will be evaluated by dose-response experiment in vitro.

Proposed combinatorial therapies that will be pursued in Aim 2: Multiple inhibitors for the identified pathways are available. Based on their reported selectivity, safety profile and anticancer activity we have selected:

- Ruxolitinib for STAT3 modulation
- LY2228820 for P38 modulation
- LY2109761 for TGF-Beta modulation
- AT7867 for AKT-modulation

Our additional studies regarding MRs of IBC cells have also identified additional targets that enhance the activity of HDAC6 only in the presence of chemotherapy. In those studies, not covered by this grant, we have used a different computational approach to evaluate the response of IBC cells through time after exposure to Ricolinostat and chemotherapy. Interestingly those studies have suggested that proteasome inhibitors (Bortezomib), as well as mTOR inhibitors (Rapamycin), may have also synergistic anticancer activity when combined with HDAC6 inhibitors. These targets are not overlapping with the ones described above and may

also expand our repertoire of putative novel targets. However, those will not be investigated under this grant funding which will be focused to the four inhibitors described above.

Remarkably, our data have also revealed that dependency on HDAC6 function was linked to its high activity and we reasoned that additional breast cancers, other than IBCs, could present the same dependency. To investigate this possibility we calculated the HDAC6-score for ~1000 primary breast cancers with available expression profile data (BRCA-TCGA² dataset). This study revealed that, consistently with our Master Regulator analysis of resistant and sensitive cells, ~15-20% of all breast cancer (enriched in hormone receptor positive (HR+) and HER2 positive (HER+)) had HDAC6-scores higher than the average HDAC6-score of the IBCs which suggests that these tumors may be sensitive to HDAC6 inhibitors (Fig. 3). An independent validation study using the Metabric³ data set containing ~2,000 primary breast cancer showed identical results (data not shown).

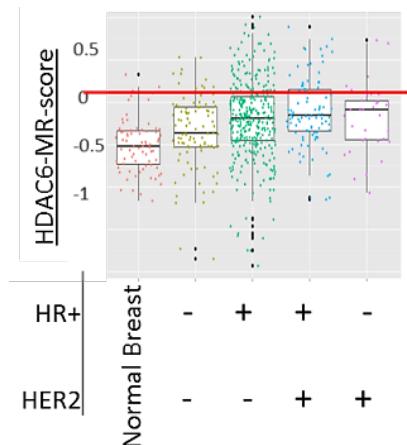


Fig. 3. Master Regulator analysis of the HDAC6-scores of primary breast cancer samples. The red line represents the average HDAC6-score for IBCs

References

- 1 Putcha, P. et al. HDAC6 activity is a non-oncogene addiction hub for inflammatory breast cancers. *Breast Cancer Res* **17**, 149, doi:10.1186/s13058-015-0658-0 (2015).
- 2 Cancer Genome Atlas, N. Comprehensive molecular portraits of human breast tumours. *Nature* **490**, 61-70, doi:10.1038/nature11412 (2012).
- 3 Curtis, C. et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* **486**, 346-352, doi:10.1038/nature10983 (2012).

4-Impact

The data that we have generated during the last year have generated two main clinically relevant findings. First we have found that HDAC6 is a master Regulator of Hormone Receptor and HER2 positive breast cancer cells and seconds that resistance to the anticancer activity of HDAC6 inhibitors is associated with activation of the STAT3 pathway. This opens the exciting opportunity of combining STAT3 inhibitors with HDAC6 inhibitors. Additionally to STAT3, other 4 signaling pathways with available inhibitors have been identified. Although STAT3 pathway emerged as the prime candidate the combined inhibition of HDAC6 with the other pathways will be also evaluated during the next period of support.

5-Changes/Problems

All specific tasks are moving forward according to the original experimental plan and no changes are proposed for the next period of support (see tables below).

Specific Aim 1(specified in proposal)	Timeline	Site 1	Site 2	Status
Specific Aim 1 tasks	Months			
1 – Investigate Novel Putative Targets (HSP-90, DNAJB12 and MEAF6).				
a) Generation and validation of shRNA and c-DNA library targeting the three selected genes	0-6	Erin Nekritz and Dr. Silva (MSSM)		Completed
b) Screens in vitro using the shRNA and c-DNA libraries in the SUM-149 cell line.	6-12	Erin Nekritz and Dr. Silva (MSSM)		Completed
c) Validation of shRNA/cDNA screens hits by lethality rescue experiments in SUM-149, Sum-190 and IBC3 cell lines	12-30	Erin Nekritz and Dr. Silva (MSSM)		In progress
d) Synergism studies for HSP-90, DNAJB12 and MEAF6 loss/gain-of-function studies combining two genes at a time (rescue experiments combination of two at a time)	24-36	Erin Nekritz and Dr. Silva (MSSM)		
e) In vitro, genome-wide level studies evaluating the consequence of inhibiting HSP-90, DNAJB12 and MEAF6 in IBC cells. These studies will consist of expression profiling followed by GSEA of SUM-149, Sum-190 and IBC3 cell lines after the three candidate genes have been knock-down by RNAi.	12-24	Erin Nekritz and Dr. Silva (MSSM)		
f) The studies from e) will be complemented by in vivo studies in the cell line SUM-149 (25 SCID mice will be used).	24-36	Erin Nekritz and Dr. Silva (MSSM)	Dr. Mundi and Dr. Califano (Columbia Un.)	
Specific Aim 2 tasks	Months			

<p>Candidate based therapy using chemotherapy plus HDAC6 inhibition.</p> <p>a) Dose-response studies with ACY-1215 in 45 breast cancer cell lines to identify sensitive vs resistant breast cancer cells.</p> <p>b) Generate expression profiles in the selected resistant and sensitive cell lines in dose-response experiment with ACY-1215.</p> <p>c) Identify Master Regulators (MRs) that define responsive vs resistant cell lines to ACY-1215 (candidate driven studies). (Phase-I)</p>	<p>0-3 3-9 9-15</p>	<p>Erin Nekritz and Dr. Silva (MSSM)</p> <p>Erin Nekritz and Dr. Silva (MSSM)</p> <p>Erin Nekritz and Dr. Silva (MSSM)</p>	<p>Dr. Mundi and Dr. Califano (Columbia Un.)</p>	<p>Completed Completed Completed</p>
<p>Evaluate combinatorial regimens HDAC6 and MRs inhibitors in preclinical in vitro. (Phase-II).</p> <p>a) MR analysis normally yields a few dozen putative candidates. Here we will utilize compound inhibitors for five of the top-ranked candidates will be evaluated by dose-response experiment in vitro in SUM-149, SUM-190 and IBC-3 cell lines as well as the resistant cell lines previously identified.</p> <p>Validation of the top candidate from a) with an additional independent inhibitor in vitro in SUM-149, SUM-190 and IBC-3 cell lines.</p>	<p>12-24 20-36</p>	<p>Erin Nekritz , Dr. Silva (MSSM)</p> <p>Erin Nekritz , Dr. Silva (MSSM)</p>	<p>Dr. Mundi and Dr. Califano (Columbia Un.)</p> <p>Dr. Mundi and Dr. Califano (Columbia Un.)</p>	<p>In Progress</p>
<p>Evaluate combinatorial regimens HDAC6 and MRs inhibitors in preclinical in vivo. (Phase-III).</p> <p>a) Obtain ACURO approval for animal work</p> <p>b) Select the top candidate for in vivo validation. Evaluation of growth inhibitory response of orthotopic xenograft mouse model of SUM-149 (we will use 10 SCID mice) when treated with small molecule</p>	<p>0-6 24-30</p>	<p>Erin Nekritz , Dr. Silva (MSSM)</p> <p>Erin Nekritz , Dr. Silva (MSSM)</p>	<p>Dr. Mundi and Dr. Califano (Columbia Un.)</p> <p>Dr. Mundi and Dr. Califano (Columbia</p>	<p>Completed</p>

<p>inhibitor for the selected candidate.</p> <p>c) Evaluation of growth inhibitory response of orthotopic xenograft mouse model of SUM-149 (we will use 15 SCID mice) when combinatorial therapeutic regiments containing chemotherapy plus the small molecule inhibitor for the selected candidate.</p>	30-36	<p>Erin Nekritz, Dr. Silva (MSSM)</p>	<p>Un.) Dr. Mundi and Dr. Califano (Columbia Un.)</p>	
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6- Products

N/A

7- Participants & other Collaborating Organizations

-Jose Silva: No Change.
-Erin Nekritz: No Change.
-Prabhjot S. Mundi: No Change.

8-Spetial Reporting Requirements

N/A